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The organisation of spinoparabrachial neurons in the mouse

Darren Cameron^{1*}, Maria Gutierrez-Mecinas^{1*}, Maria Gomez-Lima¹, Masahiko Watanabe², Erika Polgár¹ and Andrew J. Todd¹

¹Institute of Neuroscience and Psychology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom; ²Department of Anatomy, Hokkaido University School of Medicine, Sapporo 060-8638, Japan

*equal contribution

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Correspondence to: A.J. Todd or E Polgár, Spinal Cord Group, West Medical Building, University of Glasgow, University Avenue, Glasgow G12 8QQ, U.K.

AJT: Phone (+44) 141 330 5868, Fax (+44) 141 330 2868, email: andrew.todd@glasgow.ac.uk

EP: Phone (+44) 141 330 4184, Fax (+44) 141 330 2868, email: erika.beresford-polgar@glasgow.ac.uk

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ABSTRACT

The anterolateral tract (ALT), which originates from neurons in lamina I and the deep dorsal horn, represents a major ascending output through which nociceptive information is transmitted to brain areas involved in pain perception. Although there is detailed quantitative information concerning the ALT in the rat, much less is known about this system in the mouse, which is increasingly being used for studies of spinal pain mechanisms because of the availability of genetically modified lines. The aim of this study was therefore to determine the extent to which information about the ALT in the rat can be extrapolated to the mouse. Our results suggest that as in the rat, most lamina I ALT projection neurons in the lumbar enlargement can be retrogradely labelled from the lateral parabrachial area, that the great majority of these cells (~90%) express the neurokinin 1 receptor (NK1r), and that these are larger than other NK1r-expressing neurons in this lamina. This means that many lamina I spinoparabrachial cells can be identified in NK1r-immunostained sections from animals that have not received retrograde tracer injections. However, we also observed certain species differences, in particular we found that many spinoparabrachial cells in lamina III-IV lack the NK1r, meaning that they cannot be identified based solely on expression of this receptor. We also provide evidence that the vast majority of spinoparabrachial cells are glutamatergic, and that some express substance P. These findings will be important for studies designed to unravel the complex neuronal circuitry that underlies spinal pain processing.

Introduction

Nociceptive, thermal and pruritic information is conveyed from spinal cord to brain through the anterolateral tract (ALT) [50]. Cells of origin of the ALT are concentrated in lamina I and scattered throughout the deeper laminae (III-VI). Their supraspinal targets include thalamus, periaqueductal grey matter (PAG), lateral parabrachial area (LPb) and certain medullary nuclei. Individual ALT neurons can send axon collaterals to several of these regions [2,22,30,41,47].

Quantitative studies in rat lumbar enlargement have demonstrated that ALT projection cells account for ~5% of lamina I neurons. Virtually all of these can be retrogradely labelled from LPb, and they can therefore be classified as spinoparabrachial neurons [2,41,47]. Studies in rat suggest that the vast majority of lamina I spinoparabrachial neurons respond to noxious stimuli [5,7]. The neurokinin 1 receptor (NK1r), which is present at high levels in lamina I [8,10,25,55], is expressed by ~70-80% of lamina I ALT neurons in rat [4,15,50], and by a population of lamina III-IV projection neurons with dorsal dendrites that arborise in the superficial laminae [32,52]. NK1r-expressing projection neurons in laminae I and III-IV are densely innervated by nociceptive primary afferents that contain substance P (SP) [24,32,53]. Ablation of NK1r⁺ neurons by intrathecal injection of SP-saporin conjugate results in dramatic reduction of hyperalgesia in chronic pain states [28,34], which is thought to result from destruction of NK1r-expressing ALT neurons [50]. Although the NK1r is also expressed by excitatory interneurons in lamina I [25], these are significantly smaller than the projection cells [3,11]. This means that NK1r-expressing projection neurons in rat can generally be identified based on size, thus avoiding the need for retrograde tracing experiments in many cases. We have also identified a population of giant lamina I projection cells that generally lack the NK1r and are characterised by the high density of excitatory and inhibitory synapses on their cell bodies and dendritic trees [35,43].

The mouse is increasingly used for investigations of spinal pain mechanisms, but little is known about the ALT in this species [14]. The present study therefore compared the organisation of the mouse spinoparabrachial projection to that of the rat. We aimed to determine the number of lamina

I spinoparabrachial neurons, the proportion that expressed NK1r, and whether, as in rat [3], these could be distinguished from other NK1r⁺ neurons based on soma size. We also tested whether giant lamina I cells in mouse belong to the spinoparabrachial tract. In preliminary studies we found that NK1r-immunoreactive cells were infrequent in lamina III-IV of the mouse, so we tested whether spinoparabrachial neurons were present in these laminae. We have shown that the somatostatin receptor sst_{2A} is virtually restricted to GABA-immunoreactive (inhibitory) neurons in laminae I-II [37,54], however, Gamboa-Esteves et al. [18] detected sst_{2A} on some lamina I projection neurons in rat. We therefore tested for sst_{2A} expression by lamina I spinoparabrachial neurons and used anterograde tracing to assess the whether any of these cells were GABAergic. Finally, we looked for expression of neuropeptides, which has been reported for spinoparabrachial cells in other species [9,48].

Methods

Animals

All experiments were approved by the Animal Welfare and Ethical Review Board of the University of Glasgow and were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

Six adult C57Bl/6 mice of either sex (22-35g; Biological Services, University of Glasgow) were used in this study. They were anaesthetised with isoflurane and placed in a stereotaxic or spinal frame, after which anaesthetic was administered through a mask attached to the frame. In four cases, a burr hole was made through the skull, and a single injection of 200 or 300 nl of 1% cholera toxin B subunit (CTb) was targeted on the left LPb. In the other two cases, the lumbar vertebral column was exposed, and two injections of 150 nl of 1% CTb were made into the dorsal horn on one side of the spinal cord at a depth of 300 µm below the pial surface, through the intervertebral spaces rostral and caudal to the T13 vertebra. In all cases, injections were made through a glass micropipette, which was left in place for 5 minutes after completion of the injection, in order to

minimise leakage of tracer back up the track. All animals made an uneventful recovery from anaesthesia. After a 4 or 5 day survival period, they were re-anaesthetised with pentobarbitone (20 mg i.p.) and perfused through the heart with a fixative that contained 4% freshly depolymerised formaldehyde.

The brains and lumbar spinal cords were removed from all animals and post-fixed for at least 4 hours, before being cut into sections with a vibrating blade microtome.

General features of tissue processing and analysis

From all 6 mice, the region of brain or spinal cord containing the injection site was cut into transverse sections (100 μ m for brain, 60 μ m for spinal cord), and injection sites were revealed by incubating the sections in goat antibody against CTb at 1:50,000-200,000 and performing an immunoperoxidase reaction, as described previously [1,47].

The L4 spinal cord segments from the LPb-injected animals were cut into 2 series of 60 μ m thick transverse sections (each consisting of alternating sections), while the L5 segment and either L2 or L3 were cut into 60 μ m horizontal sections. The part of the brainstem that included the LPb from the spinal-injected mice were cut into 4 series of 60 μ m transverse (coronal) sections. The sections were reacted for immunofluorescence staining, by incubating them for 3 days in mixtures of primary antibodies, and then in mixtures of species-specific secondary antibodies that were raised in donkey. The secondary antibodies were conjugated to Alexa 488, Alexa 647, Rhodamine Red, Pacific Blue or biotin (Jackson ImmunoResearch) and were used at 1:500, apart from those conjugated to Pacific Blue (1:200) or Rhodamine Red (1:100). Biotinylated secondary antibodies were revealed with avidin-Pacific Blue (1:1000; Life Technologies). Following immunoreaction, the sections were mounted in anti-fade medium and stored at -20°C. All antibodies were diluted in PBS that contained 0.3% Triton-X100 and 5% normal donkey serum. The sources and dilutions of the primary antibodies are shown in Table 1.

Sections reacted for immunofluorescence were scanned with a Zeiss LSM710 confocal

microscope equipped with Argon multi-line, 405 nm diode, 561 nm solid state and 633 nm HeNe lasers, and a spectral detection system. Confocal image stacks were acquired by scanning through a 40× or 63× oil-immersion lens (NA 1.3 or 1.4, respectively) with the aperture set to 1 Airy unit. Scans through the spinal cord were scanned at a z-spacing of 1 or 2 µm, while those from the LPb were scanned at 0.5 µm z-spacing.

Quantification of CTb-labelled lamina I neurons and expression of NK1 and sst_{2A} receptors

One series of transverse sections from the L4 segment of the mice that had received injections into the LPb was reacted with antibodies against CTb (raised in mouse), NK1r and sst_{2A}. Seven sections were selected from each animal, before the CTb immunoreactivity was viewed. Two overlapping fields were scanned to include the whole superficial dorsal horn through the full thickness of the section, and these scans were analysed with Neurolucida for Confocal software (MBF Bioscience). The outline of the dorsal horn contralateral to the LPb injection was initially drawn, together with the ventral border of lamina I, which could be identified due to the plexus of NK1r-immunoreactive dendrites within the lamina. The channel representing CTb was visualised, and the locations of CTb-labelled cells were plotted onto these drawings. In order to avoid overcounting cells that were split by sectioning [20], we included cells if at least part of the nucleus (revealed as a filling defect in the CTb) was present in the first optical section in the z-series, and excluded them if part of the nucleus was present in the last optical section [1,41,47]. Because the analysis was performed on an alternate series of sections, it would not be possible for the same cell to be counted a second time on another section. The NK1r and sst_{2A} channels were then viewed, and the presence or absence of each of these types of immunoreactivity was noted for each of the CTb-labelled cells in the sample. In addition, a search was made for all lamina I cells that were immunoreactive for both NK1r and sst_{2A}, and these were then examined to see whether they were CTb-labelled.

Analysis of soma sizes for NK1r neurons and identification of giant cells

Horizontal sections from two segments (L5 and either L2 or L3) of each of the 4 LPb-injected mice were reacted with antibodies against CTb (mouse), NK1r, VGLUT2 and VGAT.

The L5 sections were used to assess the soma sizes of NK1r-immunoreactive lamina I neurons [3]. The sections that contained numerous retrogradely labelled lamina I neurons on the side contralateral to the LPb injection were initially identified, and in this way either one or two sections were selected from each animal. These were then scanned through the 40× lens with the confocal microscope to generate overlapping z-stacks (2 µm z-separation) that included the whole of lamina I apart from its lateral part, which was not analysed due to the differing orientation of cells in this region [3]. The scans were analysed with Neurolucida for Confocal, such that only the channel representing NK1r-immunoreactivity was initially visible. The ventral border of lamina I could be readily identified, because the density of NK1r-immunoreactive cells is very low in lamina II. All NK1r⁺ cells with a soma that was entirely contained within the section were identified and drawn, and the cross-sectional area of the soma in the horizontal plane was measured from the drawings [3,43]. The channel representing CTb was then viewed, and the presence or absence of retrogradely transported CTb was noted for each of the NK1r⁺ cells.

Sections from the two segments that had been cut into horizontal sections (L5 and either L2 or L3) were examined to look for giant lamina I cells, which can be recognised by the high density of VGLUT2- and VGAT-immunoreactive boutons that outline their cell bodies and dendrites [35,43]. These were initially identified by viewing the rhodamine channel (corresponding to VGLUT2), and then confirming the presence of numerous VGAT-immunoreactive boutons. Confocal z-stacks were then scanned to include the soma and proximal dendrites, and the presence or absence of CTb and NK1r was noted for each cell.

Projection neurons in lamina III-IV

Retrogradely labelled neurons in lamina III and IV were examined in the second series of transverse sections of L4 from the 4 mice that received LPb injections. These sections were reacted with antibodies against CTb (mouse antibody), NK1r, calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY). This reaction was performed because the NK1r-immunoreactive anterolateral tract cells in laminae III and IV of the rat spinal cord are densely innervated by both peptidergic (CGRP⁺) primary afferents [32], and NPY-containing inhibitory interneurons [39,40]. The sections were initially viewed with fluorescence microscopy, and those that contained CTb-labelled cells in laminae III-IV on the side contralateral to the LPb injection were identified. These cells were then scanned with the confocal microscope through the 40× lens to generate z-stacks (2 μm z-separation) through the cell body and as much of the dendritic tree as was visible. In each case, the presence or absence of NK1r immunoreactivity was noted, and we determined whether the cell body and dendrites were associated with clusters of CGRP- and/or NPY-immunoreactive axons.

Anterograde tracing

One series of sections through the brainstem of the 2 mice that received spinal injections of CTb was reacted with antibodies against CTb (mouse antibody), VGAT and VGLUT2. A single section through the LPb that contained numerous CTb-labelled boutons was selected for analysis, and scanned with an oil-immersion lens to produce z-series of 25 or 30 optical sections at 0.5 μm z-spacing. Overlapping scans were obtained from the entire region that contained CTb-labelled boutons in this section. The resulting z-stacks were viewed with Neurolucida for Confocal, and a 5 μm × 5 μm grid was superimposed on the confocal images. A single optical section within the z-stack was chosen before VGAT or VGLUT2 immunostaining were viewed, and CTb-labelled boutons were sampled by selecting the bouton nearest the bottom left hand corner of successive grid squares, starting near the dorsal- and lateral-most part of the LPb that contained these boutons and

progressing through the grid squares in a dorsal-ventral and then lateral-medial direction until the entire region containing CTb boutons had been covered [45]. The numbers of boutons analysed in the 2 mice were 100 and 208.

Another series of sections was used to investigate neuropeptide expression by anterogradely labelled terminals in the LPb. The sections were reacted with antibodies against CTb (goat antibody), enkephalin, dynorphin B and SP. From each animal, two sections that contained numerous CTb-labelled boutons were selected and scanned as described above. Again, CTb⁺ boutons were selected before the other types of immunostaining were viewed, and the selected boutons were then examined to look for neuropeptide immunoreactivity. In order to compensate for any bias towards larger profiles in the selection process [20] we measured the z-axis lengths of each of the selected boutons by determining the number of z-sections on which it was present [21,45]. The numbers of boutons analysed in this part of the study were 280 and 391 for the 2 animals.

Antibody characterisation

Specificity of the CTb antibodies is demonstrated by the lack of staining in regions that did not contain injected or transported tracer. The NK1r antibody, raised against amino acids 393-407 of the rat NK1r, recognises a 46 kDa band in Western blots of rat brain extracts, and it has been shown that there is no staining with this antibody in mice in which the NK1r has been deleted [42]. The sst_{2A} antibody was raised against the C terminal 15 amino acids of the mouse receptor, conjugated to keyhole limpet haemocyanin (KLH), and staining is abolished by pre-incubation of this peptide (manufacturer's specification). The CGRP antibody detects both α and β forms of the peptide. The NPY antibody was raised against synthetic NPY conjugated to bovine thyroglobulin, and is reported to show negligible cross-reactivity with a range of other peptides, including enkephalins, somatostatin, SP, angiotensin or vasoactive intestinal polypeptide (manufacturer's specification). The VGLUT2 antibody was raised against a synthetic peptide from rat VGLUT2, and we have shown that it stains identical structures to a well-characterised rabbit anti-VGLUT2 [51]. The

VGAT antibody was raised against amino acids 31-112 of the mouse protein, and recognises a single band of the appropriate size on Western blots [31]. The monoclonal SP antibody detects the C-terminal 5-8 amino acids of the peptide [10], and does not appear to recognise neurokinin B [29,38]. The dynorphin B antibody was raised against the full peptide, and does not cross-react with enkephalins [19]. Staining with this antibody is absent in the spinal cord of preprodynorphin knockout mice [23]. The monoclonal antibody against enkephalin recognises both Met- and Leu-enkephalin, and shows significant cross-reactivity with C-terminal extended Met-enkephalin hexa- and heptapeptides, but not with either beta-endorphin or dynorphin (manufacturer's specification).

Statistics

The Mann-Whitney U-test was used to compare the soma areas of retrogradely-labelled and non-labelled NK1r-immunoreactive lamina I neurons. Differences in the z-axis lengths of CTb-labelled boutons in the LPb that were tested for neuropeptide immunoreactivity were compared by using a *t*-test.

Results

Injection sites

Injection sites in the 6 mice are illustrated in Fig 1. In all 4 mice that received CTb injections into the brainstem, the immunoperoxidase reaction product filled the entire LPb, which extends from 1.16 to 1.88 mm caudal to the interaural plane [17] (Fig 1a,b). The injection sites also included most of the medial parabrachial area and Kolliker-Fuse nucleus and the caudalmost part of the PAG in all cases. There was variable spread into surrounding areas, including the cuneiform nucleus and cerebellum. The spinal cord injections were located in the L3-4 segments, and in both cases these filled most of laminae I-III of the ipsilateral dorsal horn (Fig 1c-e) at the levels of the injection.

Lamina I spinoparabrachial neurons: quantification and expression of NK1r and sst_{2A} receptor

Altogether, 276 CTb⁺ lamina I neurons (range 66-75, mean 69) were identified in the transverse sections of the L4 segments that were analysed from the 4 LPb-injected mice (7 sections per mouse; Table 2). This corresponds to a mean of 9.86 cells in each 60 μ m thick section, and since we have estimated that the length of the L4 segment in the mouse is 1.45 mm [37], this suggests that there would have been an average of 238 spinoparabrachial lamina I cells on the contralateral side in the entire L4 segment in these animals. There are ~4500 lamina I neurons on each side in this segment in the mouse [37], and so we estimate that approximately 5.3% of these cells belong to the spinoparabrachial tract.

As reported previously, immunoreactivity for both NK1r and sst_{2A} receptor was present on cell bodies and dendrites in lamina I, and these were found on largely separate neuronal populations, although a few cells were immunoreactive for both receptors [37]. The great majority of the CTb-labelled lamina I cells were NK1r-immunoreactive (Fig 2), with the proportion varying between 79.4-93.9% among the 4 mice (mean 89.1%) (Table 2). Sst_{2A}-immunoreactivity was found on 26 of the CTb-labelled cells (between 4-10 per mouse), and this corresponded to between 5.3-15.1% (mean 9.5%) of the retrogradely labelled neurons (Table 2). All but 2 of the sst_{2A}-immunoreactive CTb-labelled lamina I cells were also NK1r⁺. When the NK1r and sst_{2A} channels were viewed, it was found that all of the lamina I cells that were immunoreactive for both receptors were retrogradely labelled with CTb.

Soma sizes of NK1r⁺ lamina I neurons

Between 187-279 (mean 245.8) NK1r-immunoreactive lamina I cells were identified and analysed in the horizontal sections through the contralateral sides of the L5 segments of the 4 LPb-injected mice, and an example of the immunostaining for NK1r and CTb is shown in Fig 3a-c. The soma cross-sectional areas of the NK1r⁺ cells ranged from 52-1084 μ m², and showed a bimodal

distribution, with a major peak between 50-180 μm^2 , a smaller peak between 180-400 μm^2 , and a few cells with larger areas (Fig 3d). Between 17.3-25.2% (mean 20.2%) of the NK1r-immunoreactive neurons were retrogradely labelled with CTb. There was a clear difference in the soma areas of the NK1r cells that were retrogradely labelled with CTb (range 116-1084 μm^2 , median 228 μm^2) and those that were not (range 52-625 μm^2 , median 101 μm^2), and this was highly significant ($P < 0.0001$; Mann-Whitney U-test). The great majority (763/785, 97%) of NK1r cells with soma areas of less than 180 μm^2 were not retrogradely labelled with CTb, whereas most (158/198, 80%) retrogradely labelled neurons had soma areas larger than 180 μm^2 . If a soma area of 200 μm^2 was taken as a cut-off value, then 140 (71%) of the retrogradely labelled cells would be included, but only 8 (1%) of the non-retrogradely labelled cells. In other words 95% of the NK1r cells with soma areas $\geq 200 \mu\text{m}^2$ were retrogradely labelled from the LPb in these experiments.

Giant cells

Altogether, 25 giant cells were identified in the horizontal sections from the 4 LPb-injected mice (3-8 cells per animal). In each case, the cell received numerous contacts from both VGLUT2- and VGAT-immunoreactive boutons. These boutons outlined the dendritic tree, and were also numerous on the cell body (Fig 4a,e). However, unlike the situation in the rat, in which virtually all of the giant cells can be retrogradely labelled from the LPb [43], only 8 of these cells (32%, between 0 and 3 in each mouse) were labelled with CTb (Fig 4). As in the rat, we found that most of the giant cells (16/25, 64%) lacked the NK1r, although interestingly, all of the CTb-labelled cells (together with one of the cells that lacked CTb) were NK1r-immunoreactive (Fig 4).

Spinoparabrachial neurons in laminae III-IV

Retrogradely labelled neurons in lamina III-IV were counted in transverse sections of the L4 segment from each of the 4 mice that had received an injection of CTb into the LPb. This analysis was carried out on sections that had been reacted with antibodies against CTb, NK1r, CGRP and

NPY, and between 11-13 sections were examined for each mouse (Table 3). Altogether, 18 CTb-labelled cells (range 3-6, mean 4.5) were found in the contralateral dorsal horn in these laminae. Eight of these cells were NK1r-immunoreactive (Table 3), but these generally showed only weak or moderate immunoreactivity. Because of the small numbers found in each animal, we calculated the mean proportion that were NK1r-immunoreactive (8/18, 44%) for the entire sample. All 18 of these cells were associated with dense clusters of both CGRP-immunoreactive and NPY-immunoreactive boutons, which contacted their cell bodies, as well as their dendrites when these were visible (Fig. 5). The total number of lamina III-IV spinoparabrachial cells in the contralateral part of the L4 segment was estimated by dividing the rostrocaudal length of spinal cord examined (number of sections \times 60 μ m) into the total length of the segment (1.45 mm) [37] and multiplying this by the number of cells identified in the sections analysed. This gave a mean value of ~9 cells in the segment on the side contralateral to the LPb injection.

During the course of this analysis, we also observed a few NK1r-immunoreactive cells in this region that were associated with CGRP and NPY axons, but were not CTb-labelled, as well as occasional clusters of CGRP and NPY axons that were not associated with cells that were either NK1r⁺ or CTb-labelled.

Anterograde tracing

Following injection of CTb into the lumbar dorsal horn, CTb-immunoreactive boutons were present in large numbers in the LPb (Fig 6a,b), in particular in the superior, central, dorsal and internal subnuclei [17]. However, little or no anterograde labelling was seen in the external or ventral parts of the LPb, or in the Köliker-Fuse nucleus or the medial parabrachial area. This distribution is generally similar to that reported in the corresponding regions of the rat LPb following injection of anterograde tracers into the lumbar spinal dorsal horn [16,46].

The vast majority of the CTb-labelled boutons identified in the sections reacted for VGLUT2 and VGAT (95% and 99% in the 2 mice, mean 97%) were VGLUT2-immunoreactive (Fig 6c-f),

while only 3% and 0.5% (mean 2%) were VGAT-immunoreactive. The remainder (2% and 1%, mean 1.5%) were not immunoreactive for either transporter.

The results of peptide analysis are shown in Table 4, and an example of the immunostaining is shown in Fig 7. Although the majority of CTb-labelled boutons did not show any type of peptide immunoreactivity, 16% were SP-immunoreactive, with much smaller proportions (1-2%) showing dynorphin- or enkephalin-immunoreactivity. Very few of the CTb-labelled boutons showed more than one type of peptide immunoreactivity: altogether we found 3 CTb-labelled boutons that were immunoreactive for SP and dynorphin and one that was immunoreactive for SP and enkephalin.

Discussion

The main findings of this study are that: (1) spinoparabrachial cells account for ~5% of lamina I neurons in mouse lumbar enlargement; (2) ~90% of these cells are NK1r⁺, and they are generally larger than other NK1r⁺ neurons in this lamina; (3) only a minority of giant lamina I cells are labelled from the LPb; (4) many lamina III-IV spinoparabrachial neurons lack the NK1r, but as in the rat, they are innervated by both CGRP- and NPY-containing axons; and (5) spinoparabrachial axons originating from the superficial dorsal horn are nearly all glutamatergic, with some expressing SP.

Identification of lamina I ALT neurons in the mouse

By injecting different tracers into LPb and the other known major targets of lamina I cells, we have shown that in the rat, virtually all lamina I ALT neurons can be retrogradely labelled from the contralateral LPb [2,41,47]. We also demonstrated that among NK1r-immunoreactive neurons in this lamina, the great majority of the large cells could be identified as spinoparabrachial neurons, without the need for retrograde tracing experiments [3]. This raises three questions concerning the present study: (1) did we label all lamina I spinoparabrachial neurons? (2) can most lamina I projection neurons be retrogradely labelled from an injection centred on LPb? (3) can soma size be

used to distinguish between projection and non-projection cells among those that are NK1r-immunoreactive?

The LPb injection sites in our experiments included those regions that receive spinoparabrachial inputs [16,46], and numbers of retrogradely-labelled lamina I were highly consistent across the 4 mice. Wang et al [56] reported that very large injections of Fluorogold (500 nl of 4% Fluorogold) into the LPb in mice resulted in ~100 retrogradely labelled lamina I neurons per 500 μm of lumbar spinal cord, which is somewhat higher than our estimate of 9.86 cells per 60 μm (equivalent to 82 cells/500 μm). However, Wang et al included cells both contralateral and ipsilateral to the LPb injection, which probably accounts for the slightly higher cell counts that they obtained. In fact, we have shown that if two different tracers are injected into the right and left LPb in the rat, most lamina I neurons labelled from the ipsilateral LPb are also labelled from the contralateral side, indicating that lamina I spinoparabrachial neurons project either contralaterally or bilaterally [47]. It is therefore likely that we labelled virtually all lamina I spinoparabrachial neurons in the present study. Since our aim was to label the maximum number of ALT neurons, we used relatively large injections of CTb that extended into adjacent regions including the caudal part of the PAG, which also receives collaterals from lamina I ALT neurons [50]. More restricted injections would be needed to assess the proportion of these cells that actually project to LPb.

In order to test whether, as in the rat, most lamina I ALT cells are labelled from an injection into the LPb, it would be necessary to combine this with injections of a different tracer into all other known targets of these cells [2,41,47]. However, our finding that the vast majority of large NK1r⁺ lamina I neurons (soma area >200 μm^2) were retrogradely labelled strongly suggests that this is also true for the mouse. The alternative explanation would be that there was a significant population of ALT projection neurons that did not send collaterals to LPb and that this consisted mainly of small NK1r-expressing neurons or cells that lacked the receptor. Another indirect piece of evidence in support of this hypothesis is that the LPb injections labelled ~5% of contralateral lamina I neurons, which is very similar to the proportion of neurons in this lamina that belong to the ALT in the rat

[47].

The great majority of lamina I spinoparabrachial cells (~90%) expressed the NK1r, and this proportion was even higher than that seen in the rat, in which 70-80% of spinoparabrachial neurons in this lamina are NK1r-immunoreactive [2,4,15,47,52]. As in the rat [3], there was a highly significant size difference between retrogradely labelled and non-labelled cells. For example, 95% of NK1r⁺ cells with soma areas >200 μm^2 were CTb-labelled in the present series of experiments. This means that although immunostaining for the NK1r does not allow unequivocal identification of all lamina I projection neurons, it can be used to identify the larger NK1r-expressing spinoparabrachial cells with a reasonable degree of confidence, and without the need for retrograde tracing experiments.

Differences between mouse and rat

We found two major species differences in the organisation of projection neurons. Firstly the proportion of lamina I giant cells that could be labelled from the LPb, and secondly the lack of NK1r on many lamina III-IV spinoparabrachial neurons in the mouse.

Giant lamina I cells in the rat form a sparse, but distinctive population, that differ from NK1r-expressing projection neurons in their synaptic inputs and responses to noxious stimuli. They can readily be identified by the very high density of VGLUT2⁺ and VGAT⁺ boutons that almost completely outline their cell bodies and dendrites [35]. They also differ from the NK1r⁺ cells in that they receive very few contacts from peptidergic primary afferents and show significantly less expression of the transcription factor Fos after noxious thermal stimulation [35]. In the rat, we found that nearly all of these cells were retrogradely labelled from the LPb, and that they account for ~3% of spinoparabrachial cells in this lamina [35,43,50]. Examination of horizontal sections in the mouse revealed a similar population of large lamina I neurons that were coated with VGLUT2⁺ and VGAT⁺ boutons. However, only around one-third of these cells were retrogradely labelled. It is possible that the unlabelled giant cells were not projection neurons, but an alternative explanation is

that they project to other brain regions, and this will need to be assessed in future studies.

The large NK1r-immunoreactive cells in lamina III-IV of the rat form another very distinctive and easily recognised population, which was identified in early studies of NK1r distribution [8,10,25-27,34]. These cells, which respond to noxious stimuli [36], were subsequently shown to be projection neurons, because >90% could be retrogradely labelled from the caudal ventrolateral medulla, and ~65% from LPb [52]. In the rat, they receive a highly selective synaptic input from two different populations of axons: peptidergic primary afferents, which contain both SP and CGRP [32], and NPY-containing axons. The latter are thought to be derived from local inhibitory interneurons, and account for one-third of the inhibitory synaptic input [39,40]. The present study shows that cells of this type are also present in the mouse and that they appear to have the same pattern of input from peptidergic primary afferents and NPY-containing interneurons. However, many of them lack the NK1r, or express it at low levels. Immunostaining for the receptor cannot therefore be used to reveal this population or to investigate their synaptic inputs, as has been done in the rat [6,32,33,39].

The neurochemical phenotype of lamina I projection neurons

We previously reported that sst_{2A} expression was restricted to GABAergic neurons in superficial dorsal horn of both rat and mouse [37,54]. The finding of sst_{2A} on ~15% of lamina I neurons retrogradely labelled from the nucleus of the solitary tract in rat [18] therefore raised the possibility that some projection neurons in this lamina might be GABAergic. Consistent with the findings of Gamboa-Esteves et al. [18], we found that ~10% of lamina I spinoparabrachial neurons were sst_{2A}⁺. However, nearly all of these cells also expressed the NK1r, which is thought to be restricted to excitatory (glutamatergic) neurons in this lamina [25]. We had previously identified a small population of lamina I neurons that expressed both sst_{2A} and NK1r in the mouse [37], and the present results suggest that these are all projection neurons. Our finding that 97% of spino-parabrachial axon terminals are VGLUT2-immunoreactive suggests that virtually all

spinoparabrachial cells (including those with sst_{2A}) are excitatory. Our failure to detect non-GABAergic sst_{2A}⁺ neurons [37] was probably because these constitute a very small population, corresponding to ~0.5% of all lamina I neurons, compared to 17% of neurons in this lamina that are sst_{2A}-immunoreactive.

Although Standaert et al. [48] detected both enkephalin and dynorphin in lamina I spinoparabrachial neurons, they had used colchicine to increase cytoplasmic peptide levels, and it is now known that this can cause abnormal peptide expression [13,44]. Our finding that only ~2% of spinoparabrachial terminals were immunoreactive for enkephalin or dynorphin suggests that few spinoparabrachial neurons normally express significant levels of either of these opioid peptides. However, in agreement with a previous study in the cat [9], we found that some spinoparabrachial terminals were SP-immunoreactive. Relatively little is known about SP-expressing neurons in the dorsal horn, as these contain levels of peptide that are not normally detected with immunocytochemistry. However, *in situ* hybridisation has revealed cells with preprotachykinin 1 mRNA in laminae I-II [57], and non-primary SP-containing axons are seen both here and in the lateral spinal nucleus [12,51]. Since lamina I ALT neurons can generate local axon collaterals in both of these regions [49], at least some of the SP-containing non-primary glutamatergic axons [51] may be the collaterals of lamina I projection neurons.

Conflicts of interest

The authors report no conflicts of interest.

Acknowledgments

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FIGURE LEGENDS

Fig. 1 Injection sites in the 6 mice used in this study. **a** Injection sites in the 4 experiments in which CTb was injected into the lateral parabrachial area. Each vertical column represents a single experiment. The darker shaded areas show the spread of tracer, while the pale shaded area is the superior cerebellar peduncle. Numbers to the left of the drawings correspond to the position of the section posterior to the interaural plane. Drawings are based on those of Franklin and Paxinos [16]. **b** a section through the brain from the mouse #4, corresponding to a level of ~1.5 mm caudal to the interaural plane. The CTb injection site appears as a dark area, and near the top of the image, the pipette track can be seen passing through the inferior colliculus. **c, d** drawings indicating the core of the injection site in the 2 mice that received intraspinal injections of CTb. **e** a section from the spinal cord of the mouse represented in **c**. Scale bars: **b** = 1 mm, **c** = 250 μ m. IC, inferior colliculus; KF, Kölliker-Fuse nucleus; LPb, lateral parabrachial area; MPb, medial parabrachial area; PAG, periaqueductal grey matter.

Fig. 2 Expression of NK1r and sst_{2A} by retrogradely labelled spinoparabrachial neurons in lamina I. Confocal images showing a single optical section through the superficial dorsal horn in the L4 segment, contralateral to the LPb injection site. **a** CTb labelling (red) in 4 lamina I neurons identifies these as belonging to the spinoparabrachial tract. **b, c** immunostaining of the section for the NK1r (green) and sst_{2A} (blue) shows that one of these cells (marked with an arrow) expresses both receptors, while the other 3 (asterisks) are only NK1r-immunoreactive. Scale bar = 20 μ m.

Fig. 3 NK1r-immunoreactive projection neurons in lamina I. **a-c**: Confocal images from a horizontal section through the L5 segment of one of the mice that had received an injection of CTb into the LPb. The section has been scanned to reveal CTb (green) and NK1r (magenta). Six retrogradely labelled (CTb-positive) neurons are indicated with asterisks, and

each of these is NK1r-immunoreactive. In addition, several smaller NK1r-immunoreactive cells that are not retrogradely labelled (CTb-negative) are visible, and five of these are marked with arrowheads. Projection from 5 optical sections at 2 μm z-spacing. Scale bar = 50 μm . **d**: Histograms showing the numbers of lamina I NK1r-immunoreactive cells with different soma areas. The top graph shows the results for all cells (All NK1r⁺), while the middle and lower graphs show those that were not labelled (NK1r⁺ non-retrograde), or retrogradely labelled (NK1r⁺ retrograde), respectively. The dashed line corresponds to a soma area of 180 μm^2 . Note that most of the non-retrogradely labelled neurons are smaller than this, while most retrogradely labelled cells are larger.

Fig. 4 Giant lamina I neurons seen in horizontal sections. **a-d**: Confocal images showing a giant cell that was retrogradely labelled with CTb (green) and immunoreactive for the NK1r (grey). Note that many boutons containing VGLUT2 (red) or VGAT (blue) are associated with the cell body (*), and that these surround the dendrites (indicated by arrowheads). **e-h**: Another giant cell that was not retrogradely labelled, and lacked NK1r-immunoreactivity. Again, the soma (*) and dendrites (between arrowheads) are associated with numerous VGLUT2 and VGAT boutons. The two sets of images are projections of 5 (a-d) and 6 (e-h) optical sections at 1 μm z-spacing. Scale bar = 20 μm .

Fig. 5 Innervation of a lamina III NK1r-negative projection neuron by CGRP- and NPY-containing axons. **a**: Confocal image showing part of a transverse section through a lamina III neuron that was retrogradely labelled with CTb (blue). **b-d**: The cell body is surrounded by axons that contain CGRP (red) or NPY (green). The inset in **a** shows the region through the cell body (*) stained for the NK1r, and this cell is not immunoreactive for the receptor. The images are projections of 4 optical sections at 2 μm z-spacing. Scale bar = 50 μm .

Fig. 6 VGLUT2 expression by anterogradely labelled spinoparabrachial axon terminals. **a** low-magnification image through the LPb scanned to reveal CTb (red) and with dark-field optics. The LPb lies dorsal (d) and lateral (l) to the superior cerebellar peduncle (scp). **b**: The region shown in the box in **a** scanned at high magnification (15 optical sections at 0.5 μm z-spacing) shows numerous CTb-labelled boutons. **c-f**: single optical section corresponding to the region shown in the box in **b**, scanned to reveal CTb, VGLUT2 (green) and VGAT (blue). Four CTb-labelled boutons are indicated with arrowheads. These are all VGLUT2-immunoreactive, and lack VGAT. Scale bars: **a**, 200 μm ; **b**, 50 μm ; **c-f**, 10 μm .

Fig. 7 Expression of SP by anterogradely labelled spinoparabrachial terminals. **a-b**: A single optical section through the LPb reacted to reveal CTb (magenta) and substance P (SP, green). **c**: A merged image. Several CTb-labelled axonal boutons are visible, and some of these are SP-immunoreactive (two indicated with arrowheads). Scale bar = 10 μm .

Table 1 Primary antibodies used in this study

Antibody	Species	Catalogue no	Dilution	Source
CTb	mouse	ab35988	1:5,000	Abcam
CTb	goat	703	1:5,000 1:50,000-200,000*	List Biological
NK1r	rabbit	S8305	1:2K	Sigma Aldrich
sst _{2A}	guinea pig	SS870	1:2K	Gramsch Laboratories
CGRP	guinea pig	T-5027	1:10K	Bachem
NPY	sheep	AB1583	1:500	Millipore
VGLUT2	guinea pig	AB2251	1:5K	Millipore
VGAT	goat		1:1K	M Watanabe
SP	rat	OBT06435	1:200	Oxford Biotech
Dynorphin B	rabbit		1:1K	P Ciofi
Enkephalin	mouse	MAB350	1:1K	Millipore

*rabbit anti-CTb was used at 1:50-200K for immunoperoxidase staining of injection sites

Table 2. NK1r and sst_{2A} receptor immunoreactivity on CTb labelled lamina I neurons

Mouse	Total CTb ⁺ cells	% NK1r ⁺ /sst _{2A} ⁻	% NK1r ⁺ /sst _{2A} ⁺	% NK1r ⁻ /sst _{2A} ⁺
1	68	75%	4.4%	1.5%
2	66	80.3%	13.6%	1.5%
3	67	80.6%	11.9%	0%
4	75	85.3%	5.3%	0%
Mean	69	80.3%	8.8%	0.7%

The second column shows the total number of CTb-labelled cells that were identified in the 7 transverse sections analysed from each of the 4 mice. Columns 3-5 show the proportion of these cells with different receptor expression patterns.

Table 3. NK1r receptor immunoreactivity on CTb labelled lamina III-IV neurons

Mouse	Total CTb ⁺ cells	Number with NK1r ⁺	Number of sections analysed	Estimated number in L4 segment
1	6	2	11	13.18
2	3	2	12	6.04
3	5	1	13	9.29
4	4	3	11	8.79
Mean	4.5	2		9.33

The estimate of total number of lamina III-IV spinoparabrachial in the L4 segment, shown in the 5th column, was obtained by dividing the average length of the segment (1450 μ m) by the length sampled (number of sections analysed \times 60 μ m) and multiplying this by the total number of CTb cells seen.

Table 4. Neuropeptide expression in anterogradely labelled spinoparabrachial boutons

Mouse	Number of CTb boutons	% SP	% dynorphin	% enkephalin
1	280	19.6%	2.5%	0.7%
2	391	13.0%	1.5%	0.5%
Mean		16.3%	2.0%	0.6%













